A Simplified Lysis Method Allowing the Use of Biotinylated Probes in Colony Hybridization

A method is described for the lysis of bacterial cells grown on nitrocellulose filters which allows the use of nonradioactive (biotin labeled) probes in colony hybridization. Used in conjunction with a colorimetric assay involving streptavidin and alkaline phosphatase this lysis method allows the detection of clones containing a target nucleic acid sequence. Sites of positive hybridization produce dark blue–purple signals, while nonreacting clones give very light blue signals. The occurrence of false-positive and background signals is minimal. With pBR322 as the target sequence it was possible to detect approximately 20 clones in the presence of 10^5 plasmid-free cells. It was also possible to detect low-frequency plasmid-free cells in a population of clones containing the target sequence.

KEY WORDS: colony hybridization; streptavidin–biotin; nonradioactive probe technology; nucleic acid detection.

Homology-driven hybridization methods are powerful and widely used tools for the specific detection of target nucleic acid sequences. Radioactively labeled nucleic acid probes have been traditionally used in this capacity. However, because of the high waste disposal costs, short half-lives, long autoradiographic exposures, and potential health hazards associated with radioisotopes there is interest in alternative methods for the detection of target nucleic acid sequences. One such method involves the use of biotinylated nucleic acids as hybridization probes and the determination of the degree of hybridization-associated retention of biotin by the samples. The detection step can involve the use of antibiotin antibodies or employ a colorimetric reaction involving a high-affinity biotin-binding protein such as avidin or streptavidin.

In a typical procedure (1) alkaline phosphatase is polymerized to form aggregates. Biotin is then covalently attached to these aggregates. Following hybridization, filters are exposed to avidin or streptavidin, which become bound at the sites where biotin-labeled probe has hybridized to the filters. Because these biotin binding proteins are multidentate, subsequent exposure to the biotinylated alkaline phosphatase polymer results in the immobilization of phosphatase on the filters at the sites of positive hybridization. The filter-bound enzyme is then detected by a colorimetric reaction.

This protocol was originally described (1) for use in situations where purified DNA was either spotted onto the filters (Bio-blots) or transferred there from electrophoretic gels (Southern protocol) (2). We have developed and briefly described (3) a lysis method which allows the use of biotinylated probes in the detection of target sequences among arrays of organisms grown and lysed directly on filters, i.e., in colony hybridization (4). This allows the rapid screening of large numbers of clones to detect individuals harboring a target sequence. We have recently determined that the lysis protocol can be significantly abbreviated without compromising its performance. The simplified method, which

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combines the colony hybridization protocol of Grunstein and Hogness (4) with the use of biotinylated probes, is described here, along with further details on the conduct and capabilities of this method.

MATERIALS AND METHODS

Materials. Formamide was deionized by treatment with a mixed-bed ion-exchange resin (5) and stored in aliquots at −80°C. Herring sperm DNA (Boehringer Mannheim)[2] was sheared and denatured as described (5). Proteinase K was obtained from Beckman. Nitrocellulose filters, 82-mm diam, BA 85, (Schleicher & Schuell) were used throughout. Cellulose filters (Whatman 541) gave unacceptably diffuse colony patterns after lysis.

BiodUTP[3] and reagents for nick translation and for the detection of filter-bound BiodUTP were obtained from Bethesda Research Laboratories and used according to the manufacturer's directions. Bovine serum albumin was obtained from Sigma Chemical Co. (Fraction V, No. A-4503).

Filter preparation, cell growth. Filters were prepared and inoculated as described (4). Sterile filters were sealed in a plastic bag (Sears Seal-N-Save apparatus using Seal-A-Meal bags) and stored at 4°C prior to use. Cell densities of less than or equal to approximately 800 cells per filter are compatible with single colony discrimination after hybridization and color assay.

When the recovery of viable cells after the lysis–hybridization–color assay scheme was desired, replica plates were made just prior to lysis by inverting and gently laying each filter on solid growth media. Care was taken to ensure that each cell contacted the plate. Excessive pressure was not applied to the filters since this increased background in the color assay.

Escherichia coli grew to optimal size during a 14-h incubation on rich media (LB) (5) at 30°C. Cells were lysed when approximately the size of a period. For *E. coli*, larger clones gave good patterns. With more mucoid strains, such as *Xanthomonas*, the lysis of oversize cells resulted in smeared colony patterns.

Cell lysis. To lyse cells and affix their DNA to filters the following incubations were conducted (manipulations were at room temperature unless otherwise noted):

(i) 7 min, colony side up, on filter paper sheets saturated with fresh 0.5 N NaOH.

(ii) 7 min, colony side up, on filter paper sheets saturated with 0.5 M Tris–Cl, pH 8.0.

(iii) 5 min in 1.5 M sodium chloride, 0.5 M Tris–Cl, pH 7.4, 30 ml/filter. Steps (iii) through (v) and (ix) were conducted in glass petri dishes, one dish per filter. It has not been determined whether these steps could be done batchwise.

(iv) 1 h in prewarmed 200 μg/ml proteinase K in 0.15 M NaCl, 0.015 M Na citrate, pH 7.2 (SSC), 30 ml/filter, 37°C.

(v) 2 × 2 min in 90% (w/w) ethanol, 30 ml/filter.

(vi) Air-dry (20 min).

(vii) Chloroform (100 ml) was passed through each filter using the filtration device described by Grunstein and Hogness (4) (available from Schleicher & Schuell).

(viii) Air-dry.

(ix) 5 min, 0.3 M NaCl, 30 ml/filter.

(x) The filters were individually sandwiched between filter paper, wrapped loosely in aluminum foil, and baked at 80°C *in vacuo* for 2 h.

(xi) Storage: vacuum desiccator.

A maximum of 12 filters was processed at a time.

[2] Mention of a particular product does not imply recommendation by the U.S. Department of Agriculture over similar items of equal performance.

[3] Abbreviations used: BiodUTP, biotin-11-deoxyuridine-5′-triphosphate; BCIP, 5-bromo-4-chloro-3-indolylphosphate; NBT, nitroblue tetrazolium.

Gentle suction was applied to the filters after steps (i) through (iv) by means of a slab gel dryer and water aspirator. This reduced the dispersion of cells from the sites of growth, promoting tighter patterns. A single sheet of filter paper was used as an underfilter. A rubber sheet (A. H. Thomas No. 8095D40) into which six filter-size holes had been cut was laid atop this to serve as a template for the aspiration of six filters at a time.

Prehybridization, hybridization, color assay. Prehybridization buffer (1,6) contained 300 μg of sheared, heat-denatured herring sperm DNA per milliliter and was 50% (v/v) in formamide. This mixture was made up in bulk, stored at −20°C, and discarded after one use. Pairs of filters were enclosed back-to-back in Seal-A-Meal bags and incubated 2 h at 42°C in 20 ml of prehybridization solution.

Purified DNA was prepared by detergent lysis of bacteria and isopycnic banding in the presence of ethidium bromide (5). DNA was biotinylated by replacing dTTP with BiodUTP in a nick-translation reaction catalyzed by *E. coli* DNA polymerase I. Amplification of the input DNA was not achieved by this method. Unincorporated BiodUTP was removed by chromatography on Sephadex G-50 (6).

Biotinylated probe DNAs were denatured prior to hybridization by incubating for 10 min in a boiling water bath and quick-chilling on ice. Hybridization was conducted at 42°C in buffer (1,6) containing 45% formamide and 190 ng biotinylated DNA/ml. Sodium dextran sulfate could be omitted from the hybridization buffer without ill effect. A 1-h hybridization was sufficient for the detection of unamplified pBR322. A 15-h hybridization was required to detect a 18-MDa *Xanthomonas campestris* plasmid under investigation in this laboratory. Hybridization solutions were recovered after use, pooled, and stored at −20°C. They could be reused at least 10 times over a time span of at least 5 months without a noticeable reduction in performance. Probes were heat denatured before each use.

Posthybridization washes were conducted as described (6). The use of Fraction V albumin resulted in acceptable results. Fatty acid-free albumin was found to interfere with the detection assay.

The filter-bound probe was detected by a procedure employing streptavidin and alkaline phosphatase (6). Briefly, the filters were sequentially exposed to streptavidin and biotinylated alkaline phosphatase. This causes the immobilization of alkaline phosphatase at sites of positive hybridization on the filters. Filters were then incubated with BCIP and NBT. Indoxyl generated from BCIP by the action of alkaline phosphatase condenses to form indigo (blue). Indigo then reacts with NBT to form insoluble diformazan (purple). Color development was conducted in dim light, monitored visually, and terminated when reacting colonies were intensely purple. Filters were stored moist in sealed bags. Elapsed time from the end of hybridization to the termination of color development was approximately 3 h.

RESULTS

This lysis method allows the use of biotinylated nucleic acids as probes in a colony hybridization format to differentiate between cells harboring and cells lacking a target nucleic acid sequence. Cells containing sequences homologous to the probe yield an intense blue–purple color while nonreactive clones remain colorless or light blue. Nonspecific false-positive background reactions are negligible.

Figure 1 illustrates the use of this method to identify *E. coli* harboring plasmid pBR322. Plasmid-free and plasmid-containing cells were inoculated onto filters lying on growth media. Following growth and lysis of the cells the filters were probed with biotinylated pBR322 as described above. Upon application of the biotin-specific color assay plasmid-free clones gave a very light purple color (Fig. 1A). These clones could be readily differentiated from those harboring the plasmid (Fig. 1B), which gave very dark blue–

(A)

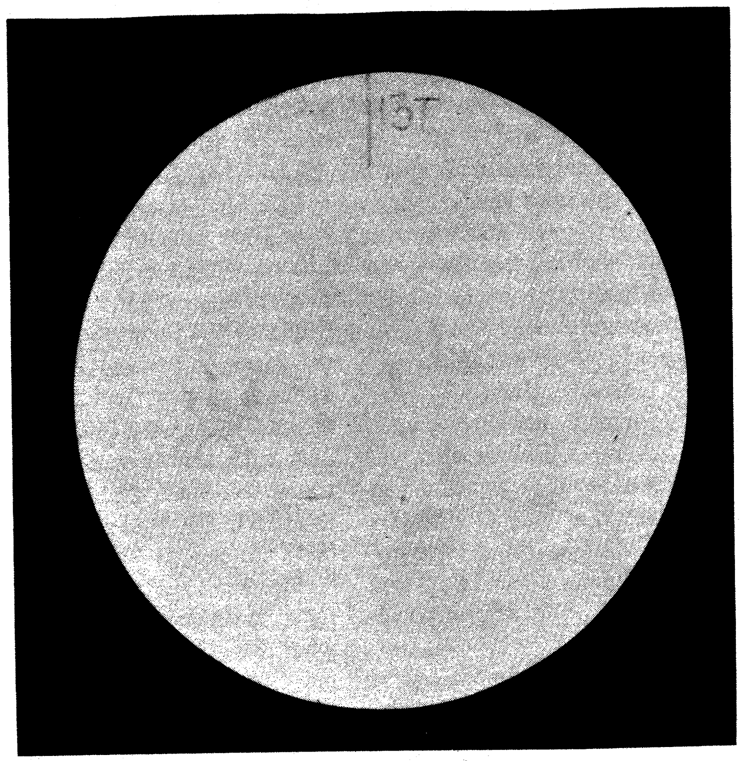

FIG. 1. Specific identification of *E. coli*-containing plasmid pBR322 by colony hybridization. Cell growth, lysis, DNA fixation to the filters, hybridization and the detection of filter-bound probe were as described. The hybridization probe was biotinylated pBR322. (A) Plasmid-free *E. coli* C600r^-m^-. (B) *E. coli* RR1 containing pBR322. (C) A 10 to 1 mixture of the plasmid-free and plasmid-containing cells. Approximately 225 clones were present on each filter.

purple signals. Cells containing the target sequence could be detected in the presence of a 10-fold excess of cells lacking sequences homologous with the probe (Fig. 1C). The "tailing" of color away from each clone is associated with the use of gentle swirling during the lysis. We have recently determined that mixing is not required during the lysis and that it has this detrimental effect on resolution.

It is possible to detect target cells when they represent a much smaller proportion of the total cell population than is shown in Fig. 1C. To determine the detection limit, plasmid-free cells were inoculated at several densities onto nitrocellulose filters. Small volumes of a dilute culture of *E. coli* RR1/pBR322 were then applied to specific sites on the filters. The extent of dilution was such that fewer than 15 colonies were deposited at each inoculation site. The filters were incubated until confluent growth occurred, treated to lyse the cells, and incubated with biotinylated pBR322 in hybridization solution. The sites of positive hybridization were determined by color assay. It was possible to detect positively reacting cells, visible as small dark dots, in the presence of vast excesses of nonreacting ones. There was absolute correlation between the locations of the dark spots on these filters and the sites where cells bearing pBR322 had been spotted. In Fig. 2, approximately 25 positively reacting clones, spread among three inoculation sites on a filter containing 10^5 plasmid-free cells, can be identified. It was possible to detect plasmid-containing cells in the presence of

(B)

(C)

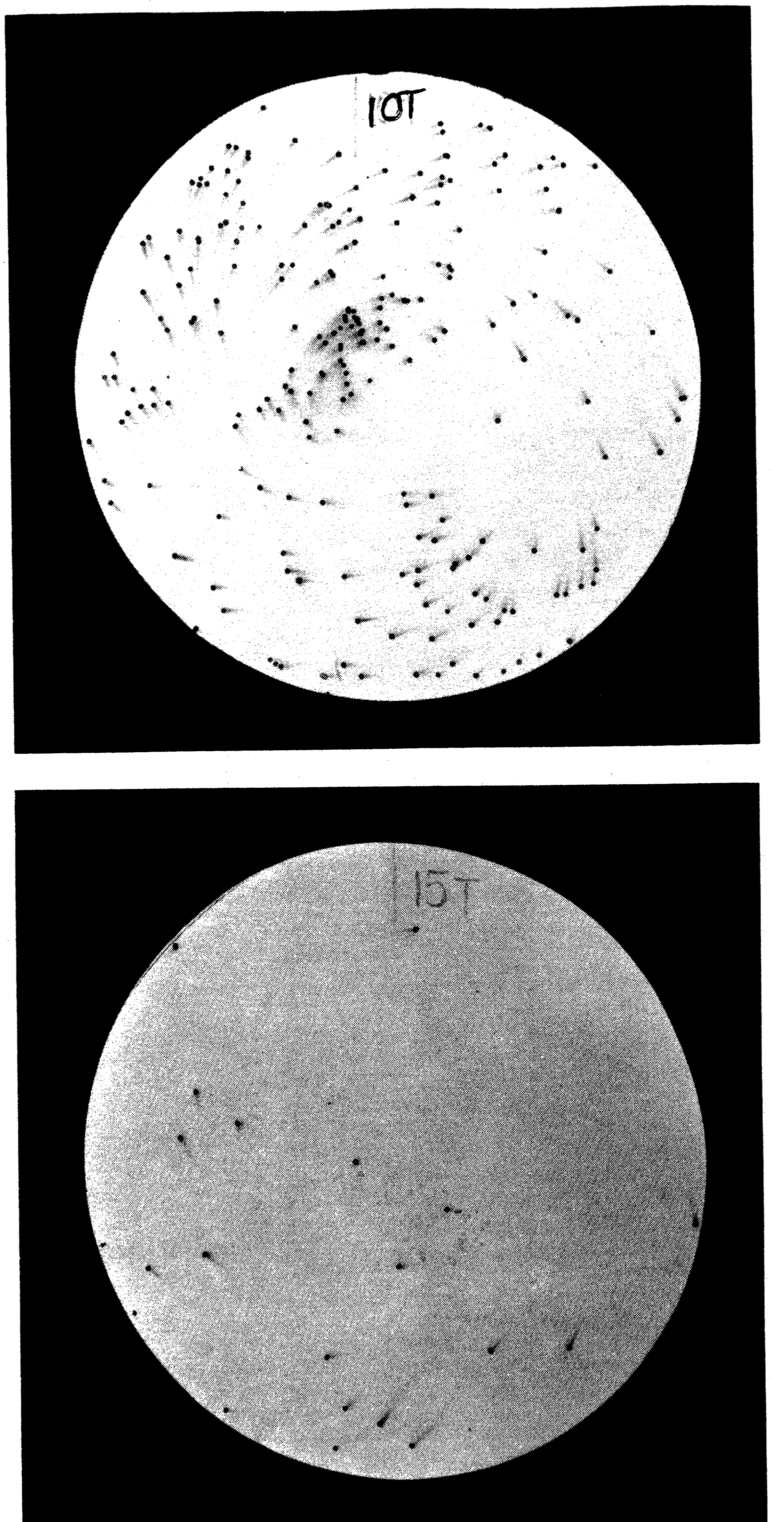

FIG. 1—*Continued.*

even higher numbers of nonreactive cells (e.g., 10^6), but the signal strength was low in this case.

It was of interest to determine if one could successfully scan a cell population for rare individuals lacking a particular DNA sequence. This could aid, for example, in the detection of cured cells in a population harboring a cryptic plasmid. *E. coli* RR1/pBR322 was spread on a filter at moderate density (2300 cells). A small portion of the filter was left uninoculated. Into this space was placed a volume of dilute, plasmid-free *E. coli* sufficient to produce one to four clones. After cell growth, lysis, hybridization with biotinylated pBR322, and determination of the sites of positive hybridization, the only spots which were not deeply purple were those corresponding to the locations at which plasmid-free cells had been inoculated (Fig. 3, arrow A). The density of positively reacting sites on the filter in Fig. 3 would have interfered with the detection of a few cured cells randomly dispersed among the population. However, it is clear that even at this cell density the plasmid-free cells are distinctly lighter than the rest of the population. There is no appreciable transfer of positively reacting material from cells harboring the target sequence to those lacking it. Streaks of color are located near some of the positively reacting clones shown in Fig. 3 (arrow B). These are easily distinguished from negatively reacting clones and do not interfere with the identification of cells lacking the target plasmid. Thus this method can be used

FIG. 2. Ultra high-density screening: the detection of low frequency cells harboring a target sequence. Strains, methods, and probe were as described for Fig. 1. The arrows indicate the sites at which cells containing the target sequence were inoculated on the filter, and where positive hybridization was in all cases localized. Inocolum: 10^5 cells, approximately 25 of which contained the target sequence, per filter.

FIG. 3. Detection of plasmid-free cells in a population of cells containing the target plasmid. A volume of *E. coli* RR1/pBR322 sufficient to yield approximately 2300 clones was spread uniformly on a filter, leaving a small uninoculated region just beneath the identification number of the filter. Into this open region was placed a volume of plasmid-free *E. coli* C600 sufficient to yield between one and three clones. After cell growth the filters were processed as above. Arrow A indicates the location of light colored plasmid-free cells. The dark spots covering the rest of the filter correspond to the locations at which plasmid-containing cells grew. Arrow B indicates faintly reacting material which originated from the positive clones and did not correspond to actual clones on the filter.

for the detection of low-frequency individuals lacking a target sequence.

DISCUSSION

The method described here is significantly shorter and easier to perform than our original protocol (3). Treatments with lysozyme and organic solvents have been omitted. Twelve filters can now be processed in 5 h, as opposed to the 7 h required for the previous method. The qualities of the patterns obtained by the two lysis methods are comparable. We have recently determined that steps (ii) and (ix) can also be omitted without negative effect. The remaining steps are all required in order to obtain strong signals at positively reacting sites and acceptably low background color. Since treatment with phenol has been omitted from the revised lysis method, nylon filters may now be useful in this application.

Our detection method involved incubation of the filters, after hybridization, with streptavidin, alkaline phosphatase, and the phosphatase detection reagents NBT and BCIP. (Results were the same whether the streptavidin was covalently linked to the alkaline phosphatase or noncovalently joined by binding to biotin residues previously attached to the phosphatase.) Due to the affinity of streptavidin for biotin, alkaline phosphatase subsequently became immobilized on the filters at the sites of positive hybridization. The final color development was conducted under dim light since the reactants are light sensitive. It was necessary to observe the development of color at frequent intervals (10 min) and to stop the reaction when positively reacting colonies were deep purple. Incubation past this point caused the color of nonreacting clones to darken such that they were mistaken for positives. Overdevelopment is the greatest single factor contributing to the appearance of false-positive signals.

The ethanol concentration in step (v) of the lysis protocol is weight per weight. Ethanol solutions made up volume per volume, or otherwise in excess of 90% w/w, exceed the ethanol tolerance limits of some batches of nitrocellulose. Filters washed in such solutions may become brittle and be reduced nearly to powder by the end of the hybridization–color assay protocol. The appropriate solution can be made from 100% ethanol.

Another feature of this method which was not detailed in our initial report is that it allows the detection of target cells among a vast excess of cells not harboring the sequence of interest (Fig. 2). This represents some of the highest density screening ever reported. Single colony resolution and direct recovery is impossible at these densities. However, high-density screening can be used in a qualitative mode to determine the presence of low-frequency sequences in a cell population. Also, by recovering from replica plates the areas corresponding to a positive signal it is possible to recover a mixture of cells, some of which contain the target sequence. This mixture can be diluted, grown on filters, and reprobed to identify cells harboring the sequence of interest.

We have not rigorously determined the minimum probe concentration necessary to obtain strong positive hybridization signals. We have noted, however, that probe concentrations of 10–20 ng/ml, in conjunction with overnight hybridizations, are too low to give strong hybridization signals for nonreiterated target DNAs 3 MDa or larger in size.

The data presented here describe the use of pBR322 as probe for the detection of sequences in *E. coli.* We have also achieved suitable results using other probes and organisms, including the use of an 18-MDa plasmid of *X. campestris* to probe for homologous sequences in this mucoid organism and the use of DNA of the fungus *Rhizopus delemar* to detect sequences cloned from this organism into *E. coli.*

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